Repetitive, Marker-Free, Site-Specific Integration as a Novel Tool for Multiple Chromosomal Integration of DNA

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We present a tool for repetitive, marker-free, site-specific integration in *Lactococcus lactis,* in which a nonreplicating plasmid vector (pKV6) carrying a phage attachment site (*attP*) can be integrated into a bacterial attachment site (*attB*). The novelty of the tool described here is the inclusion of a minimal bacterial attachment site (*attB$_{min}$*), two mutated *loxP* sequences (*lox66* and *lox71*) allowing for removal of undesirable vector elements (antibiotic resistance marker), and a counterselection marker (*oroP*) for selection of *loxP* recombination on the pKV6 vector. When transformed into *L. lactis* expressing the phage TP901-1 integrase, pKV6 integrates with high frequency into the chromosome, where it is flanked by *attL* and *attR* hybrid attachment sites. After expression of Cre recombinase from a plasmid that is not able to replicate in *L. lactis,* *loxP* recombinants can be selected for by using 5-fluoroorotic acid. The introduced *attB$_{min}$* site can subsequently be used for a second round of integration. To examine if *attP* recombination was specific to the *attB* site, integration was performed in strains containing the *attB*, *attL*, and *attR* sites or the *attL* and *attR* sites only. Only *attP*-*attB* recombination was observed when all three sites were present. In the absence of the *attB* site, a low frequency of *attP*-*attL* recombination was observed. To demonstrate the functionality of the system, the xylose utilization genes (*xylABR* and *xylT*) from *L. lactis* strain KF147 were integrated into the chromosome of *L. lactis* strain MG1363 in two steps.

L actic acid bacteria are industrially important microorganisms with widespread applications in the dairy industry. In addition, they show great potential as cell factories for production of a range of products, including food ingredients (1, 2) and pharmaceutical agents (3). Consequently, tools for genetic manipulation to insert novel genes or pathways are of great interest. Numerous classical tools for insertion of genes are already available (4–8); however, none of these allow for iterative integration cycles. In some cases, the procedures involved are tedious and time-consuming, and in other cases, reuse is hampered by a limited number of selection markers.

A crucial factor in designing new strains is genetic stability. The presence of plasmids may lead to instability and, subsequently, loss of the plasmids. In addition, the plasmids often result in a metabolic load (9), and the plasmid copy number often varies with growth, resulting in different expression levels of the genes they carry. A strategy to avoid these problems is chromosomal integration. A site-specific recombination system for *Lactococcus lactis* that generates stable, single-copy chromosomal integration, based on the lactococcal temperate bacteriophage TP901-1, has previously been described (4). In this system, the TP901-1 integrase facilitates site-specific recombination, at a high frequency, between the two nonidentical *attB* (43 bp) and *attP* (56 bp) attachment sites, located in the chromosome of *L. lactis* MG1363 and on a constructed integration vector, respectively. When the vector integrates, hybrid *attL* and *attR* attachment sites flanking the vector are generated. Although this system is extremely useful, there are some drawbacks. The entire plasmid, including an antibiotic selection marker, is left on the chromosome after the integration event, and iterative integration is not possible.

Methods for excising markers from the chromosome do exist, and the *loxP*/Cre recombination system is one of them (10–12). If a marker is flanked by *loxP* sites oriented in the same way, Cre-mediated recombination results in excision of the marker, and a functional *loxP* site is left behind. The latter is a disadvantage when

the *loxP*/Cre system is to be reused, as the presence of multiple *loxP* sites could result in undesirable recombination events. Various versions of the *loxP*/Cre system have been developed, including the *lox66* × *lox71*/Cre system, which was developed for stable chromosomal integration. When the mutated *lox66* and *lox71* sites recombine, the outcome is one degenerate *lox72* site, which is a poor substrate for the Cre recombinase, and one normal *loxP* site (13). Using the *loxP*/Cre system with a counterselection marker would be an efficient tool for excising markers from the chromosome. Previously, we demonstrated that the *L. lactis* orotate transporter encoded by *oroP* may serve as an efficient counterselection marker when 5-fluoroorotate (5-FO) is present (5, 14). Most wild-type *L. lactis* strains are not sensitive to 5-FO, as it cannot enter the cell, but when the transporter is expressed, severe growth retardation is observed when cells are plated on defined medium with 5-FO. In this study, we designed, constructed, and tested a system that can be used for repetitive insertion of genes on the chromosome, based on a combination of the above-mentioned tools. The integration vector pKV6 carries both an *attP* site and a minimal chromosomal attachment site (*attB$_{min}$*) (15), mutated *loxP* sites flanking the multiple cloning site and the *att* sites, and the *oroP* counterselection marker. Combining all these elements facilitates easy, repetitive, marker-free, site-specific integration. The system has been used successfully to introduce genes involved in xylose metabolism into *L. lactis* MG1363 in two consecutive steps.

MATERIALS AND METHODS

Bacterial strains and plasmids. For cloning purposes, *Escherichia coli* strains MC1000 (16), ABLE C (Stratagene), and ABLE K (Stratagene) were used. *L. lactis* subsp. *cremoris* MG1363, a prophage-cured and plasmid-free derivative of NCDO712 (17), was used as the host for site-specific integration in the chromosomal *attB* site of the lactococcal phage TP901-1. MG1363 was transformed in advance with either pLB65 or pLB95, both harboring the *int* gene, encoding the TP901-1 integrase (4), to facilitate site-specific integration. The plasmid pLB65 confers resistance to chloramphenicol, whereas pLB95 carries a temperature-sensitive origin of replication and confers resistance to tetracycline (4). MG1363 with pLB65 was used for testing 5-fluoroorotate sensitivity and for characterization of the specificity of the site-specific recombination event. MG1363 with pLB95 (LB504 [4]) was applied for testing the system for repetitive, marker-free, site-specific integration. *L. lactis* subsp. *lactis* KF147 (27) chromosomal DNA was used as the template for amplification of the xylose utilization genes *xylABRT* and as a control strain in screening for growth with xylose as the sole carbon source. *L. lactis* subsp. *lactis* IL1403 was used as the host for site-specific integration, as it contains a region that resembles the bacterial attachment site for the TP901-1 integrase (81.4% homology to the $attB_{min}$ sequence). The plasmid pCS1966 (5) was used as the template for construction of the integration vector pKV6. The plasmid harbors the *attP* site from phage TP901-1 for site-specific integration; the *ermAB* genes, conferring resistance to erythromycin; the *oroP* gene, conferring 5-FO sensitivity; and an *E. coli* origin of replication. The plasmid pBluescript KSII (Stratagene) was used as the template for amplification of the *bla* gene inserted into pKV6. Plasmid pLB85 and its derivative pCS574 were applied for investigation of the direction of integration. Both plasmids carry the *attP* site; however, pLB85 confers erythromycin resistance (4), while pCS574 confers tetracycline resistance (30). For determination of transformation efficiency, competent cells were transformed with pGhost8 (6).

Cell growth and antibiotics. *E. coli* strains were grown aerobically at 37°C (MC1000) or 28°C (ABLE C and ABLE K) in modified LB (10 g liter^{-1} peptone from casein, 5 g liter^{-1} yeast extract, 4 g liter^{-1} NaCl) with appropriate selective antibiotics. *L. lactis* strains were cultivated at 28°C when pLB95 was present, and otherwise at 30°C, without aeration, in M17 broth (18) or chemically defined SA medium (19) modified by exclusion of acetate and addition of 2 μg ml^{-1} lipoic acid (SAL). The media were supplemented with 1% (wt/vol) glucose, giving GM17 and GSAL, respectively. Xylose was added to either 0.2% or 2% (wt/vol) xylose. The following concentrations of antibiotics were used: ampicillin, 100 μg ml^{-1} for *E. coli*; erythromycin, 5 μg ml^{-1} for *L. lactis* and 150 μg ml^{-1} for *E. coli*; tetracycline, 5 μg ml^{-1} for *L. lactis* and 8 μg ml^{-1} for *E. coli*; and 5-FO, 50 μg ml^{-1}. The pLB95 plasmid was cured as previously described (6).

DNA manipulations. Unless stated otherwise, standard procedures were used for DNA manipulation and *E. coli* transformation (20). Chromosomal DNA from *L. lactis* was isolated as described for *E. coli*, with the addition that the cells were treated with 20 mg ml^{-1} of lysozyme for 2 h before lysis. *L. lactis* cells were made electrocompetent, and DNA was introduced by electroporation as previously described (21). However, for transformation with pKV7, GSAL medium supplemented with 20 mM sucrose was added directly after transformation and incubated for 2 h. Cells were washed with 0.9% NaCl before dilution and plating on GSAL supplemented with 50 μg ml^{-1} 5-FO. PCR amplification procedures were performed using either DreamTaq DNA polymerase, for analytical purposes, or Phusion DNA polymerase, for cloning and sequencing (Fermentas). Oligonucleotides were ordered from either Sigma-Aldrich or Integrated DNA Technologies. DNA fragments were purified with a GFX PCR DNA and gel band purification kit (GE Healthcare). Plasmid DNA was isolated with a Zyppy plasmid miniprep kit (Zymo Research). FastDigest restriction enzymes and T4 DNA ligase were obtained from Fermentas.

Construction of plasmids. For the construction of pKV6, *lox71* and *lox66* sites were inserted into pCS1966 by amplifying the plasmid with 5′-phosphorylated primers, with one of the primers carrying the *loxP*

TABLE 1 Primers used in this study

Primer	Sequence (5′–3′)^a
pCS699	CTAGT CTAGA TTAAA AAATA AAAAA GAACC CACTC GGGTT CTTTT TTCTG ATAAT TGCCA ACACA ATTAA CATCT CAATC AAGGT AAATG CAACA ACCTT GTATC AAAGG T
pCS700	GCTCT AGAAC TAGTG GATC
p5	[P]-TACCG TTCGT ATAGC ATACA TTATA CGAAG TTATA TAGCT GTTTC CTGTG TG
p6	[P]-ACGCT GTTCC ATATT ACTAG
p7	[P]-ATAAC TTCGT ATAGC ATACA TTATA CGAAC GGTAG AGCTC CCTTC TATGC ATG
p8	[P]-ACGCT GTTCC ATATT ACTAG
p10	GTTGT GTGGA ATTGT GAG
p11	AACTA CTGCT GCTTC ACC
p12	GGAAG AAAGC TTTGG CAA
p16	GTTTT TCTTT TTGTG CGGAC
p18	GATAA TAATA CTGGT CCGC
p21	CCGTC TTATC TCCCA TTA
p54	CAGTG GATCC TTATCC CTCTT CTTCT AAATG
p55	CTAGC TCGAG ATTTC CTAGC CACTG CAT
p56	CAGTG GATCC TTAAC CTTCG TCATT GAA
p57	CTAGC TCGAG GAGTC TAAAA ATGAC TACTT GT
p72	AGCTA GTAA**U** ATGGA ACAGC GTATA ACT
p73	AGTGG GAA**U** TGTAC CCCTT ATCGA TA
p74	ATTTT TAA**U**G ATTAA GTTGG GTAAC GC
p75	ATTAC TAGC**U** ACATT ATAAA AATTT AAT
p76	ATTCC CAC**U**G GAACC CCTAT TTGTT TA
P77	ATTAA AAA**U**G AAGTT TTAAA TCAATCT
p91	CATTG CAGAG TTTAT TCTTG ACATA GATAG AGAAA ATTGA TATAA TAGGC GAAGT AATAA AATAT TCGGA GGAAT TTTGA AATGA CTAAG AAAGT AGCAA TCTAT
p92	[P]-GACTC ACTAT AGGGC GAA

^{a} [P] indicates 5′ phosphate. Uracil residues (U) are in bold for emphasis.

sequences. The PCR product was religated and transformed into MC1000. The *lox71* site was inserted with primers p5 and p6, and the *lox66* site with primers p7 and p8 (Table 1). The $attB_{min}$ sequence and the *groEL* terminator were inserted with primers pCS699 and pCS700. The PCR product was digested with XbaI before transformation into strain MC1000, resulting in pKV5. A novel functional *oroP* gene and the *bla* gene were inserted using a modified version of USER fusion (22). USER fusion is a PCR-based method that allows for simultaneous fusion and cloning of multiple PCR fragments. The desired fragments for cloning are amplified with PCR primers containing a single deoxyuridine residue near the 5′ end. Subsequently, the PCR fragments are treated with a commercial deoxyuridine-excision reagent which generates overhangs designed to specifically complement each other (22). In this study, the plasmid was likewise amplified by PCR. For PCR amplification, the PfuX7 polymerase (23) was applied following the standard procedure for Phusion polymerase. Plasmid pKV5 was amplified with the USER primers p72 and p73, the novel *oroP* gene was amplified from pCS1966 with the USER primers p74 and p75, and the *bla* gene was amplified from pBluescript KSII with the USER primers p76 and p77. All three PCR products were treated with DpnI and purified with GFX before being mixed at a ratio of 1:1:1. USER mix (New England BioLabs) and NEBuffer 4 (New England BioLabs) were added to final concentrations of 0.1 U μl^{-1} and 1×, respectively. The mix was incubated at 37°C for 35 min, followed by 25 min at 25°C. Ligation was performed before transformation into electrocompetent ABLE K cells. The plasmid was subsequently transformed into MG1363 harboring pLB65. Colonies were restreaked on GSAL supplemented with 50 μg ml^{-1} 5-FO for confirmation of a functional *oroP* gene.

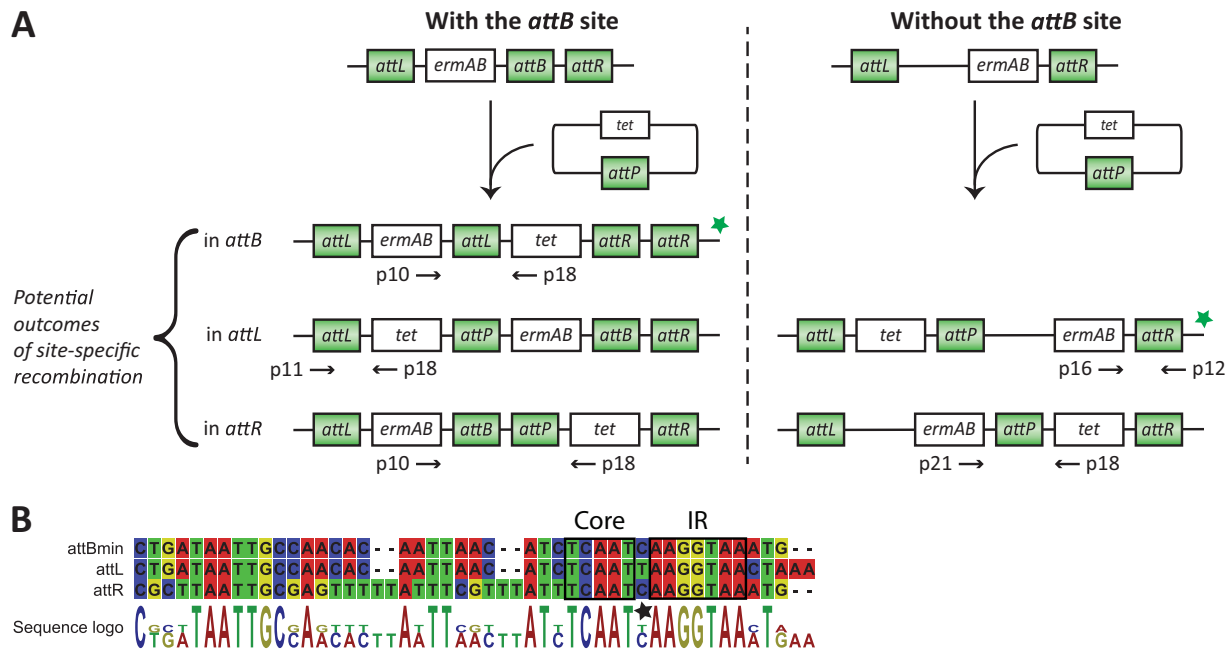The *xylABR* and *xylT* genes were inserted into pKV6, resulting in

FIG 1 Specificity of the site-specific recombination event. (A) Potential outcomes of site-specific recombination between the *attP* attachment site, located on a nonreplicating plasmid, and *attL*, *attR*, and *attB* or *attL* and *attR* only, located on the chromosome. The arrows indicate the binding sites of the primers applied in the screening for positions of integration, and the green stars indicate how integration occurred. (B) Alignment of *attB* and *attL* and part of *attR*. The black star marks a variation between the sequences that was previously shown to have no effect. The two additional differences between the *attB* and *attL* sequences are underlined in the alignment. IR, inverted repeat.

pKV9 and pKV10, respectively. The *xylABR* genes were amplified from *L. lactis* KF147 with primers p56 and p57. The *xylT* gene was amplified with primers p54 and p55. The PCR fragments were digested with BamHI and XhoI and ligated to pKV6 digested with the same enzymes. The ligation mix was transformed into electrocompetent ABLE C cells.

A codon-optimized *cre* gene was obtained from GenScript (see the supplemental material for the sequence). The *purF* promoter (TTTTT CCGAA CAATT AACTT GAAAA AATTA AAAAA GTCCT ATTAG GACTT TTTAT TTCCA AACAA TATAG ATAGA ATTAA AATAT AATAA AAGAA TCGAT AGAGG GAAAA AATT) was inserted in front of the gene. The gene and promoter were flanked by the *L. lactis groEL* (AAAAA AGAAC CCGAG TGGGT TCTTT TTTAT TTTTT AA) and *fbaA* (TTTGC TTGTT AAACT AATAT AAAAA AGAAA TTGCT ATTAA GCAGT TTCTT TTTTT TGATT TAAGA GTGCA TATTA CG) terminators. The construct was inserted by GenScript into pUC57. The resulting plasmid, pKV7, was transformed into ABLE K cells.

Characterization of the specificity of the site-specific recombination event. MG1363 harboring pLB65 was transformed with pKV6 and pLB85, resulting in strains KV8 and KV9, respectively. Selection was performed with erythromycin. Subsequently, KV8 and KV9 were transformed with pCS574, and selection was performed with erythromycin and tetracycline. The integration frequency was determined as the number of CFU per μg pCS574 DNA, and the transformation frequency as the number of CFU per μg pGhost8 DNA. In total, 70 transformants from each transformation were screened by PCR. For KV8/pCS574, primers p10 and p18 were applied for screening for integration in *attB* (487 bp) and *attR* (530 bp), and primers p11 and p18 were used for screening of integration in *attL* (301 bp). For KV9/pCS574, primers p18 and p21 were applied for screening of integration in *attR* (390 bp), and primers p12 and p16 were used for screening of integration in *attL* (191 bp). Integration in *attB* was verified by sequencing (Macrogen, The Netherlands).

TP901-1 integrase with a strong synthetic promoter. The TP901-1 integrase was amplified with a strong synthetic promoter (5′-TGCAG AGTTT ATTCT TGACA TAGAT AGAGA AAATT GATAT AATAG GC

G-3′ [−35 and −10 regions underlined]) (17) and the aldolase leader (5′-AAGTA ATAAA ATATT CGGAG GAATT TTGAA-3′) from MG1363. The primers used were p91 and p92, and the template DNA was pLB95. The PCR product was ligated and purified before transformation.

RESULTS AND DISCUSSION

Characterizing the specificity of the site-specific recombination event. When *attP* and *attB* recombine, two hybrid sites are formed: *attL* and *attR*. Since the strategy chosen for iterative integration into an *attB* site involves introduction of a new *attB* site during every cycle, it was important to determine that *attB* is indeed the preferred substrate for the integrase. Strains containing two (*attL* and *attR*) or three (*attL*, *attR*, and *attB*$_{min}$) attachment sites were constructed and subsequently transformed with pCS574, which carries *attP* (Fig. 1A). A 15 (15 ± 4.5)-fold higher integration frequency was found for the strain containing *attB*$_{min}$ than for the one with only *attL* and *attR*. The difference in the transformation efficiencies of the two strains (with and without *attB*$_{min}$) was taken into account by using a replicating plasmid (see Materials and Methods). This revealed that *attB* was indeed the preferred substrate for the integrase, but also that either *attL* or *attR*, or perhaps both, can function as integration sites.

To determine whether *attL*, *attR*, and/or an unidentified site can function as a bacterial attachment site, the chromosomal positions of the integration events were determined by PCR. Four primer sets were designed to give PCR products for each of the possible integration events in *attB*, *attL*, and *attR* (Fig. 1A). In total, 140 integrants were analyzed, including 70 in the presence of *attB* and 70 in the absence of *attB*. When *attB* was present, only *attP*-*attB* recombination was observed, whereas when *attB* was not present, integration was observed in *attL*. This confirmed that
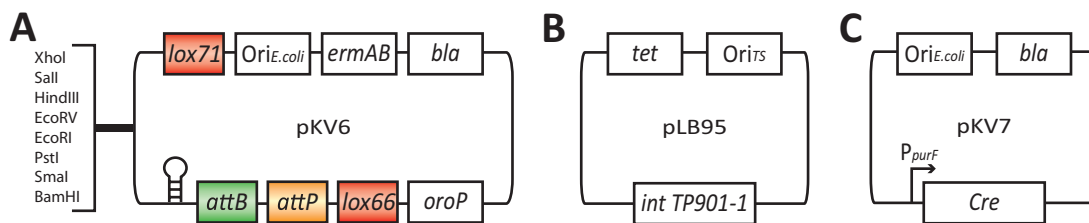
FIG 2 Plasmids applied in the repetitive, marker-free, site-specific integration system. (A) Integration vector pKV6. The *attP* site recombines with the chromosomal *attB* site. The *attB*$_{min}$ site facilitates repetitive integration. The two *lox* sites allow for removal of undesired vector elements. The *oroP* gene functions as a counterselection marker, as it encodes a dedicated orotate transporter that likewise transports the toxic analogue 5-fluoroorotate. (B) *L. lactis* replicating thermosensitive plasmid pLB95, carrying the *int* gene, encoding the TP901-1 integrase, to facilitate *attB-attP* recombination. (C) *L. lactis* nonreplicating plasmid expressing Cre recombinase, facilitating excision of undesired vector elements.

the TP901-1 integrase has a higher affinity for *attB*. No *attL* recombination was observed when *attB* was present, indicating a higher reaction rate for *attB-attP* recombination than for *attL-attP* recombination. The results clearly demonstrate that this system can be used for repetitive integration.

In the absence of *attB*, *attL* may act as an integration site, although less efficiently than *attB*. This is justified by comparing the sequences of *attL*, *attR*, and *attB*$_{min}$ (Fig. 1B). *attB*$_{min}$ and *attL* clearly display more sequence homology than *attB*$_{min}$ and *attR*. Except for two additional bases in the *attL* sequence, only three variations are present in the *attL* sequence compared to the *attB*$_{min}$ sequence. The T$_{33}$ variation in *attL* (marked with a star) has previously been shown to have no effect on the recombination frequency (15). The lower affinity of the TP901-1 integrase for *attL* may therefore be explained by the C$_{41}$ and A$_{43}$ variations (underlined in the alignment).

Construction of the integration vector pKV6 and the *cre* plasmid pKV7. Plasmid pCS1966 was used as the starting point for constructing the iterative integration vector, as it carries the *attP* site needed for site-specific integration into the chromosomal *attB* site and the *oroP* gene, encoding an orotate transporter that can be used as a counterselection marker. The minimal attachment site, *attB*$_{min}$, was inserted into the vector together with the *groEL* terminator from *L. lactis* to avoid transcriptional read-through into subsequently inserted genes. The pCS1966 plasmid harbors genes conferring resistance to erythromycin. In addition, the *bla* gene conferring resistance to ampicillin was inserted as an extra marker. The *lox66* and *lox71* sites were inserted as direct repeats. The *lox* sequences flanked the region of the plasmid with the different markers and the *E. coli* origin of replication, which was expected to be removed when the Cre recombinase was expressed. The resulting plasmid, pKV6 (Fig. 2), was constructed and maintained in *E. coli* strain ABLE K, which lowers the copy number of ColE1 high-copy-number derivatives, as high levels of the orotate transporter had a negative effect on growth of *E. coli*. When it was introduced into the chromosome of *L. lactis* MG1363, the plasmid conferred sensitivity to 5-FO.

With respect to the expression of the Cre recombinase in *L. lactis*, it has previously been shown that a small amount of Cre recombinase is sufficient to facilitate the *loxP* recombination event. In addition, a small amount of Cre recombinase gives the highest recombination efficiency (24). Thus, a weak inducible promoter, *purF*, was chosen for expression of the *cre* gene. The *purF* promoter is repressed in the presence of exogenous purines and activated in their absence (25), and thus it is active in the

minimal media applied for selection with 5-FO (5). A DNA fragment containing the *purF* promoter from *L. lactis* IL1403 driving transcription of a codon-optimized *cre* gene was ordered as a synthetic construct in the vector pUC57. To ensure good translation, the aldolase leader was included, as this leader provides efficient translation of the open reading frame (26). The plasmid, named pKV7 (Fig. 2), does not replicate in *L. lactis*, which ensures transient expression of the Cre recombinase.

Inserting the xylose genes of *L. lactis* KF147 into *L. lactis* MG1363. In order to validate the method, the xylose utilization genes from *L. lactis* KF147 were inserted into the chromosome of *L. lactis* strain MG1363 in two consecutive steps. KF147, unlike MG1363, can naturally grow on xylose. Four genes are specifically involved in metabolism of xylose in KF147: the *xylA* gene encodes a xylose isomerase, *xylB* encodes a xylulose kinase, *xylR* encodes a transcriptional regulator, and *xylT* encodes a xylose transporter. Introduction of the xylose genes was accomplished in two rounds. Each round involved TP901-1 integrase-mediated integration followed by excision of the integrated plasmid by use of Cre recombinase. The TP901-1 integrase gene was supplied on the thermosensitive plasmid pLB95 (Fig. 2) (4), which can easily be removed by a simple temperature shift, whereas the Cre recombinase was expressed transiently using the nonreplicating plasmid pKV7 described above.

In the first round, *xylABR* was introduced (Fig. 3A). The *xylABR* genes were inserted into pKV6, resulting in plasmid pKV9. The plasmid pKV9 was transformed into MG1363 already harboring the thermosensitive plasmid pLB95 (LB504). Ten integrants were tested by PCR, and all had *xylABR* inserted as expected. One of the clones (KV90) was chosen and subjected to the Cre recombination procedure to obtain a strain without markers. KV90 was transformed with pKV7 to introduce Cre activity, and the transformants were spread on GSAL medium supplemented with 5-FO to select for loss of the markers. KV90 was also plated directly on 5-FO plates without being transformed with the Cre plasmid. In both cases, 5-FO-resistant colonies appeared. As shown below, the presence of 5-FO-resistant strains which have not been subjected to Cre recombination can be explained by excision due to a certain reversibility of the TP901-1 integrase that was still expressed from pLB95 (28). Fifteen 5-FO-resistant colonies that had been subjected to Cre activity were selected for further investigation. In 10 of the 15 colonies tested, the markers flanked by the *loxP* sites had been deleted, leaving behind only *xylABR* with the mutated *lox72* site and a new *attB*$_{min}$ site flanked by *attL* and *attR* (Fig. 3A). The transient expression of Cre obtained by transforming the nonrep-
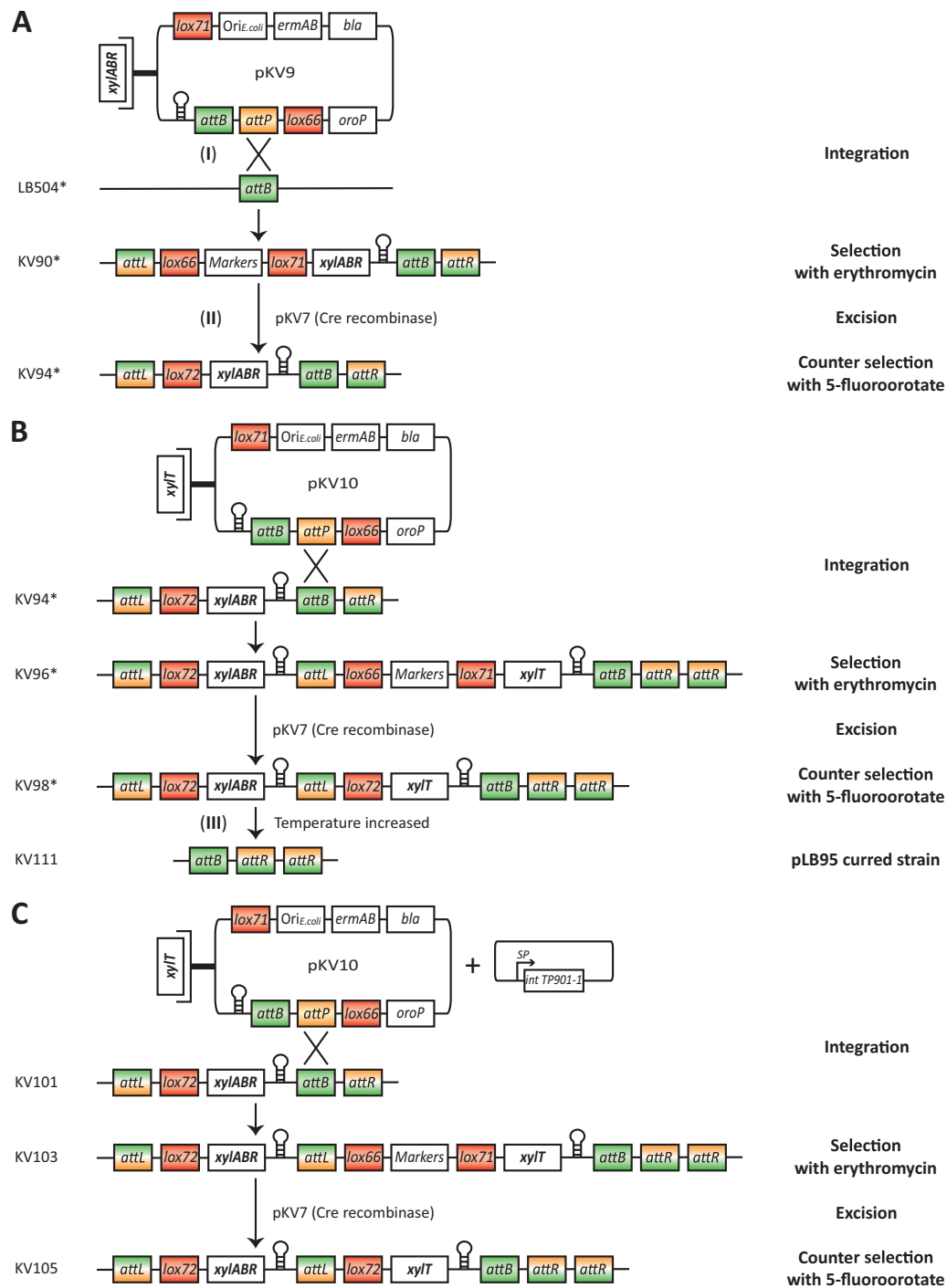
FIG 3 Introduction of xylose utilization genes into the chromosome of *L. lactis* MG1363 in two steps, using the repetitive, marker-free, site-specific integration system. Two derivatives of the integration plasmid pKV6, carrying the xylose utilization genes *xylABR* (pKV9) and *xylT* (pKV10) from *L. lactis* KF147, were used consecutively. (A) Integration of plasmid pKV9 followed by excision of unwanted elements. Integration (I) is a site-specific recombination event between the *attP* and *attB* attachment sites which results in formation of *attL* and *attR* attachment sites. Integration is mediated by the TP901-1 integrase, expressed from plasmid pLB95, with a temperature-sensitive origin. Excision (II) is mediated by the Cre recombinase, which is expressed from the nonreplicating plasmid pKV7. The substrates for the Cre recombinase are the modified *loxP* sites *lox66* and *lox71*, and the product is *lox72*, which is a poor substrate for the Cre recombinase (13). (B) The second integration and excision event, obtained using the same approach as in panel A. Following excision, the temperature was increased to facilitate loss of the helper plasmid carrying the TP901-1 integrase gene (III). However, this unexpectedly resulted in loss of the xylose genes. (C) The two-step procedure was repeated, this time using transient expression of the TP901-1 integrase from a circularized PCR product, and the result was successful integration of the xylose genes. *, strains with pLB95. LB504 is strain MG1363 harboring pLB95 (4).

licating pKV7 plasmid into *L. lactis* was thus sufficient for high-frequency recombination between the mutated *loxP* sites. For the five remaining colonies, the *attL* site and the novel *attB*$_{min}$ site had recombined, leaving only the *attB* and *attR* sites. One of the correct strains (KV94) was chosen for the next round of integration.

In the second round, *xylT* was cloned into the integration vector pKV6 (pKV10) and used to integrate *xylT* into KV94 (Fig. 3B). Among 10 integrants, 6 were shown to be correct using PCR, and 1 (KV96) was subjected to the Cre recombination procedure by transformation with pKV7 and subsequent plating on 5-FO. Five 5-FO-resistant candidates were tested by PCR, and two (KV98 and KV99) had lost the markers flanked by the two *loxP* sites but retained the *xyl* genes (Fig. 3B). They were subsequently verified by sequencing. All the *xyl* genes were now present in the MG1363 derivatives KV98 and KV99. All the markers introduced on the chromosome had been excised again, and only the previously introduced plasmid pLB95, carrying the TP901-1 integrase gene, was still present.

Continuous expression of TP901-1 integrase negatively affects the genetic stability of the inserted DNA. To obtain a marker-free strain, the pLB95 plasmid carrying the TP901-1 integrase gene had to be removed. Since the plasmid had a temperature-sensitive replication system, a simple temperature shift from 28°C to above 35°C should have facilitated this. However, when this was done for KV98 and KV99, large deletions of the inserted constructs were observed for all tested colonies. Sequencing revealed that the *attL* site from the first round of integration and the *attB*$_{min}$ site left after the second integration recombined, resulting in a strain with only the *attB*$_{min}$ site and the two *attR* sites left (Fig. 3B). The increase in temperature seemed to have unknown effects on either expression or function of the TP901-1 integrase, resulting in excision of the inserted genes. This was also observed at the temperature permissive for plasmid replication (28°C), although at a lower frequency (data not shown). Again, this could most likely be explained by infrequent excision facilitated by the TP901-1 integrase, as mentioned above. KV94 was likewise cured for pLB95, resulting in strain KV101. KV101 still had the *xylABR* genes present (verified by PCR), and the plasmid had been lost (Fig. 3C). Based on this observation, the effect of the reversibility of the TP901-1 integrase most likely increased as a second integration was performed. Clearly, another way to express the TP901-1 integrase was needed.

Expression of the TP901-1 integrase from a ligated PCR product. Since continuous expression of the TP901-1 integrase negatively affected genetic stability, the integrase should be expressed only during the integration steps. An alternative means of integrase expression is to express it from a plasmid that is not capable of replicating in *L. lactis*, a strategy which has been shown to work previously (29). We decided to try a different approach: cotransformation of the integration vector pKV6 with a PCR product containing a strong promoter driving expression of the TP901-1 integrase-encoding *int* gene. DNA harboring the *int* gene was amplified by PCR with a strong synthetic promoter and the aldolase leader from *L. lactis*, previously applied successfully to achieve high expression levels (30). Both linear and circularized PCR products were tested, the latter because linear DNA might be degraded rapidly inside *L. lactis* and thereby prevent sufficient levels of the integrase from accumulating. Indeed, high-frequency integration was observed for the circularized PCR product (4.9 × 10^4 μg^{-1}), whereas no integrants were obtained by cotransformation with linear PCR. Using this approach, the genetic instability

was avoided, and a strain (KV105) containing both *xylABR* and *xylT* without selection markers was successfully constructed (Fig. 3C). In addition, the counterselection with 5-FO was improved, as only successfully *lox66 × lox71*-recombined transformants were obtained after transformation with pKV7, and no 5-FO-resistant colonies appeared when the transformed strain was plated directly on the 5-FO plates. Overall, this new approach made the workflow more efficient.

Introducing xylose utilization genes into *L. lactis* MG1363 does not lead to growth on xylose. Although the introduction of intact *xyl* genes was demonstrated (and verified by sequencing), neither KV94, KV96, KV98, nor KV105 was able to grow on xylose. The *xylABR* genes were therefore introduced into the *L. lactis* strain IL1403. IL1403 cannot grow on xylose, even though the *xylABRT* genes are all present (31). The *attB* site found in *L. lactis* MG1363, however, is not present in IL1403. Instead, IL1403 contains a region that resembles the bacterial attachment site for the TP901-1 phage (81.4% similarity to the *attB*$_{min}$ sequence). Correct integrants in the IL1403 attachment site were obtained when IL1403 was cotransformed with pKV9 and the circularized TP901-1 *int* PCR product (verified by PCR). The introduction of the *xylABR* genes into IL1403 was sufficient to enable growth on xylose, suggesting that additional traits are needed before MG1363 can efficiently utilize xylose as the sole carbon source. In addition, this demonstrated that the tool for repetitive, marker-free, site-specific integration described here can be used in *L. lactis* strains that do not contain the *attB*$_{min}$ site but instead contain a sequence that resembles the attachment site for the TP901-1 phage. A BLAST search demonstrated that the *attB*$_{min}$ site found in MG1363 can also be found in other *L. lactis* subsp. *cremoris* strains (NZ9000, A76, and SK11), whereas the IL1403 integration site was identified in *L. lactis* subsp. *lactis* strains KF147 and IO-1.

We conclude that the tool for repetitive, marker-free, site-specific integration described here is a powerful tool for comprehensive strain construction and to obtain stably engineered strains without antibiotic markers. Martín et al. previously reported a similar system for inserting genetic material into the chromosome in lactic acid bacteria (32). With this system, it was likewise possible to integrate genes into the chromosome and subsequently remove the antibiotic marker. There are, however, two significant advantages with our system. First, the application of counterselection using 5-FO reported here is far less laborious than the screening for loss of antibiotic resistance performed by Martín et al. Second, our system can be used repeatedly. As neither the Cre recombinase nor the TP901-1 integrase requires host cofactors and both have been shown to be functional in a range of organisms, including mammalian cells (33), the principle of the system is universal and can easily be transferred to other organisms.

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